A Bacterial Test for Plant Food Accessories (Auximones).

By W. B. Bottomley, M.A., Professor of Botany, University of London, King's College.

(Communicated by Prof. F. W. Oliver, F.R.S. Received June 1, 1915.)

In a previous communication* attention was called to the significance of certain accessory food substances for normal plant growth. It was pointed out that the nutrition of a plant depends, not only upon the supply of mineral food constituents, but also upon the presence of certain accessory organic food substances, very small amounts of which are sufficient to satisfy the needs of the plant. These plant food accessories are analogous in some respects to the curative substances of beri-beri and scurvy which Suzuki calls "oryzanine," and for which Funk has suggested the name "vitamine," thinking they are of an amino nature. More recently Moore and his collaborators have applied the term "torulin" to the curative substance obtained from yeast.

Experiments in progress indicate that the plant food accessories resemble more closely the growth-stimulating food factors of Hopkins than the vitamines of Funk, and the term "auximone" (Gr. αὔξιμος, promoting growth) is suggested for them, being descriptive of their action rather than of their nature or composition, about which nothing definite is known.

Hitherto the only means of demonstrating the presence of these plant auximones has been their action on the higher plants. Unfortunately this is a long process and often unsatisfactory owing to the difficulty in maintaining constant environmental conditions during the comparatively long period of growth, and a more ready means of demonstrating their presence is desirable in order to facilitate further investigation of their constitution and properties.

The effect of the plant food accessories obtained from an alcoholic extract of bacterised peat on the growth and nitrogen fixation of *Azotobacter chroococcum*, described in a previous paper, suggested the possibility of a bacterial test for the more active fractions of the alcoholic extract. As experiments had shown that the fractions of this extract of bacterised peat obtained by means of phosphotungstic acid and by silver and baryta, according to the method already described, gave growth results with wheat plants, an investigation was made of the effect of these fractions on the growth of Azotobacter.

Eighteen flasks, each containing 100 c.c. of distilled water, 1 grm. mannite, 0·2 grm. K_2HPO_4, 0·02 grm. $MgSO_4$, and 0·2 grm. $CaCO_3$, were divided into

* 'Roy. Soc. Proc.,' B, vol. 88, pp. 237–247 (1914).

three series of six flasks each. The first series served to test the growth of the organism in normal culture. To each of the flasks of the second series was added the phosphotungstic fraction from 1 grm. of bacterised peat, and to the flasks of the third series the silver fraction from a similar quantity. The amount of dry substance thus added to each flask was 0·00017 grm. in the case of the phosphotungstic fraction, and 0·000035 grm. in the case of the silver fraction. Each flask was then inoculated with 1 c.c. of a uniform suspension of Azotobacter in distilled water, and two flasks from each series were sterilised to serve as controls. After incubation for 10 days at a temperature of 26° C., the contents of each flask were analysed by the Kjeldahl process for its nitrogen content. The results obtained are as follows:—

Table I.

Series.	Nitrogen-content.		Nitrogen fixation.	Mean nitrogen fixation.
		mgrm.	mgrm.	mgrm.
I. Normal mannite solution	1. Control	0·1		
	2. „	0·1		
	3. Culture	4·0	3·9	
	4. „	3·8	3·7	3·9
	5. „	4·0	3·9	
	6. „	4·1	4·0	
II. Normal mannite solution + phosphotungstic fraction from 1 grm. bacterised peat	1. Control	0·2		
	2. „	0·2		
	3. Culture	9·7	9·5	
	4. „	10·1	9·9	9·7
	5. „	10·0	9·8	
	6. „	9·9	9·7	
III. Normal mannite solution + silver fraction from 1 grm. bacterised peat	1. Control	0·2		
	2. „	0·2		
	3. Culture	10·3	10·1	
	4. „	10·5	10·3	10·4
	5. „	10·9	10·7	
	6. „	10·7	10·5	

Although these results were promising, the use of this method as a test for plant auximones was found to be unsuitable owing to the variability of the organism and the length of time required for incubation, and a shorter and more reliable method was desirable.

Preliminary experiments had already shown that the application of bacterised peat to the soil resulted in an increased production of nitrates, and an examination of the effect of these auximones on nitrification in the soil yielded further interesting results. Two equal quantities of soil weighing 2 lb. each were taken, and to one was added, in solution in distilled water, the phosphotungstic fraction of that weight of bacterised peat (30 grm.)

which, if incorporated with the soil, would have given a mixture of 10 parts of soil to one part of peat by volume, the proportion used in the preliminary experiments. The weight of solid matter thus introduced amounted to 0·051 grm. After small portions of each had been weighed out for analysis, the two samples of soil were put into wide-mouthed glass bottles, loosely corked, and kept at laboratory temperature for some weeks, the bottles being well shaken daily to ensure aëration, and distilled water added when necessary, to maintain a uniform moisture-content. Small samples were taken from time to time, and their nitrate-content determined by the phenol-sulphonic acid method, with the following results :—

Table II.

	Nitric nitrogen in parts per million on—				
	April 6.	April 20.	April 30.	May 12.	May 26.
Soil	11	78	95	228	316
Soil + auximone	14	153	305	471	662

These results suggested that liquid cultures of the nitrifying organisms might furnish a test for plant auximones. A culture was therefore obtained by placing 10 grm. of garden soil in a flask containing 100 c.c. tap-water, 0·1 grm. $(NH_4)_2SO_4$, 0·1 grm. K_2HPO_4 and 0·2 grm. $MgCO_3$ (Winogradsky's medium), and incubating for seven days at 26° C., at the end of which period the liquid showed a strong reaction for nitrate. Sub-cultures were then made from this liquid into fresh nitrifying solutions, and, after a further week's incubation, a second sub-culture was made, which was used for testing the effect of the auximone.

A series of eighteen flasks was then prepared, six containing normal nitrifying culture solution, six the normal solution plus phosphotungstic fraction from 1 grm. of bacterised peat, and six the normal solution with the addition of the silver fraction. All were inoculated from the second sub-culture of nitrifying organisms and incubated at 26° C. At the end of 48 hours all the flasks containing auximone showed a thick scum on the surface of the liquid, and when examined at the end of six days were found to contain no trace of nitrate, while in those flasks without auximone, where no scum had developed, nitrification had proceeded normally. Some contamination of the medium was suspected, and the work was repeated, great care being taken with the sterilisation of flasks and media. Again a scum appeared in all the liquids containing auximone, and a third experiment yielded similar results.

The constant formation of this scum whenever the auximone was added to the crude nitrifying culture from soil suggested the possibility that either the scum-forming organisms were introduced with the auximone, or the scum formation might be used as a specific test for auximones. To test this a sub-culture was made from an original soil culture and incubated for four days. No scum formed. This was then divided into two portions, one of which was autoclaved at 140° C. for half an hour, after which the phosphotungstic fraction from 1 grm. of bacterised peat was added to each, and both were re-incubated for three days at 26° C. A thick scum formed on the unsterilised liquid, but no trace appeared on the sterilised one, thus showing that the scum organisms are present in the soil culture, and the formation of the scum is due to the presence of the auximone.

An examination of the scum shows that it consists of two predominant kinds of organisms: a thin beaded-rod form and a spindle-shaped form. The nature of the scum depends upon the relative proportion of these two organisms, being crinkled and gelatinous when the beaded forms predominate, and smooth and brittle when the spindle forms are in a majority. By continuous plating out pure colonies of each of these forms were obtained, but when grown separately in nitrifying solution plus auximone the characteristic scum never appeared. Further investigations are in progress as to the identity and nature of these organisms.

The soil from which the scum-forming organisms were first obtained was a rich garden soil from Kew, and an examination of other soils was made in order to determine whether the organisms are of fairly constant occurrence or whether they are restricted to certain localities. Upwards of a dozen samples of soils, including loams, clays and gravel, from various places were tested, and all were found to yield the characteristic growth in the nitrifying solution with the addition of auximone. The rapidity of formation of the scum, however, varied considerably, the best and most rapid growth being obtained from a sample of new loam from a virgin (uncultivated) field; a very good growth from some old potting mixture; and a very slow and poor growth from soil from a bed of leguminous plants.

Although the organisms are thus found to be widely distributed and easily obtainable, it became necessary for experimental purposes to obtain a uniform stock from which a good growth could be readily obtained. Some soil was therefore sterilised, put aside for a week, and then saturated with a suspension of the scum-forming organisms. It was allowed to dry down at room temperature under sterile conditions, and stored in a bottle. This stock can be depended upon to yield a good growth of scum in from two to three days in the presence of auximones.

The scum is most readily obtained by placing about 10 grm. of soil in a nitrifying solution and incubating it for two days before adding the auximone. The first trace of scum appears in 24 hours after this addition, and increases as incubation is continued, until at the end of four to six days it becomes so thick that it sinks to the bottom of the flask, and no second scum is formed. Sub-cultures from this growth are used for test purposes. It is found, however, that successive sub-culturing from the original scum rapidly produces an alteration in the nature of the growth, very little scum being formed, and the liquid becoming turbid and bright yellow in colour. Hence the necessity for obtaining a fresh scum from the prepared soil for each new set of experiments.

In order to test whether these organisms are able to indicate the relative quantity of plant auximone present, six series of three flasks each were arranged as follows:

Series.	Nos.						
A	1–3	Contained 100 c.c. normal culture solution.					
B	4–6	,,	,,	,,	+4·2	parts per million silver fraction from bacterised peat.	
C	7–9	,,	,,	,,	+2·1	,,	,,
D	10–12	,,	,,	,,	+0·35	,,	,,
E	13–15	,,	,,	,,	+0·07	,,	,,
F	16–18	,,	,,	,,	+0·007	,,	,,

All were inoculated with the scum-forming organisms, and incubated at 26° C. After 36 hours, series A showed no trace of scum, B an extremely thick one, C a thick one, D a moderate growth, E a fair growth, but not nearly so good as D, and F no appreciable growth; hence the rate of growth and thickness of scum show a progressive increase with the quantity of auximone present above a certain minimum, which in this case was the extract from 0·2 grm. of bacterised peat.

The fact that this minimum amount, which represents only one part of the dry silver fraction in sixteen millions of culture solution, gives a formation of scum indicates the sensitiveness of the organisms to a very minute trace of auximone.

As there was a possibility of other substances present in the phosphotungstic and silver fractions being concerned in the scum formation, an investigation was made of the effect of the presence of certain organic substances in the nitrifying solution. The usual standard employed in tests with bacterised peat extract has been the phosphotungstic fraction from 1 grm. of bacterised peat per 100 c.c. of culture solution, which represents a solution of seventeen parts of dry substance per million of liquid.

Accordingly, nitrifying solutions containing seventeen parts per million of sucrose, maltose, asparagine, peptone, leucine, tyrosine, and hordein respectively, were inoculated with the organisms, but no growth occurred after four days. Fresh liquids, containing twenty, forty, and sixty parts per million of each of these substances were then tested, with negative results; and not until the proportions had been increased to two hundred parts per million in each case was any growth apparent. Even then the characteristic scum was not obtained, the whole medium becoming uniformly cloudy. This shows that the scum formation is due to the specific action of auximones.

The next step was to determine if the presence of the accessory substances concerned with animal nutrition would induce scum formation. These substances have been obtained from various seeds and yeast. For this experiment the seeds employed were wheat, maize, and peas. They were first soaked in water for 24 hours, then dried and left at room temperature for two days, when their radicles were from $\frac{1}{2}$ to 1 inch in length. They were then ground up in a mortar, and a phosphotungstic fraction obtained of these, of similar quantities of the dry seeds, and of yeast, in the same way as described for bacterised peat. The extract of 1 grm. of each of the seeds and of yeast was used for testing, and no scum could be obtained with the phosphotungstic fraction of dry maize and dry peas. A fair scum, however, was obtained in from two to three days with the fractions from yeast, germinated wheat, peas, and maize, and a thin scum from dry wheat. In the case of wheat, the seeds are invested with a pericarp, and it was from this region of rice grains that an animal food accessory was first obtained. It is thus evident that the scum-forming organisms are able to serve as a qualitative test for food accessories in general.

Having thus obtained an indicator for auximones, it becomes a comparatively simple matter to examine other materials for their presence. Since bacterised peat, in which auximones are relatively abundant, is obtained by bacterial action upon raw peat, it was decided to investigate other samples of decomposing organic matter for these substances. Quantities of fresh stable manure, and also of a well-rotted two-year-old manure, were procured and fractionated in the usual manner. Portions of these fractions, corresponding to 1, 2, 4, 6, 8, 10, 20, 40, and 50 grm. respectively, of both fresh and rotted manures were added to nitrifying solutions, three flasks being used for the investigation of each of the separate portions. After inoculation with the scum-forming organism, the whole series of flasks was incubated for four days when the first indication of a scum appeared in the liquids containing the fraction from 10 grm. of rotted manure, and in those which had received the

extract from 50 grm. of fresh manure. It would thus appear that the quantity of auximone present increases with the progressive decomposition of the organic matter of the manure; although the relatively small amount even in the two-year-old rotted manure is apparent, when it is stated that a better formation of scum was obtained with the fraction from $\frac{1}{5}$ grm. of bacterised peat than with 10 grm. of the manure.

An unexpected source of plant auximones has been discovered in the root nodules of leguminous plants. A quantity of root nodules from bean plants were collected, and the phosphotungstic fraction obtained from them in the usual way. The roots which bore the nodules were also extracted separately. On investigation it was found that a very thin film was obtained when nitrifying solutions containing the phosphotungstic fractions of $\frac{1}{40}$ grm. of roots and nodules respectively were inoculated with the scum-forming organism. Liquids containing $\frac{1}{20}$ grm. gave a fair growth, and those containing $\frac{1}{10}$ grm. a good growth, the extract from nodules giving a slightly better growth than that from the roots in each case. A similar fraction was obtained of the roots of beans which had been grown in sterilised sand and which had formed no nodules, and no growth at all could be obtained upon nitrifying solutions to which had been added the extract from $\frac{1}{10}$ grm. of such roots.

Some further interesting points may be noted in connection with this research. The organisms which form the scum require no organic carbon for their growth, and are similar to the nitrifying organisms and sulphur and iron bacteria in that they can assimilate atmospheric carbon dioxide by the process of chemosynthesis. Further they cannot live on nitrates, but must obtain their nitrogen from an ammonium salt.

The plant auximones so far investigated differ in one important respect from those concerned with animal nutrition in that they are not destroyed by heating. A phosphotungstic extract from bacterised peat gave a thick scum after being heated in an autoclave at 134° C. for half an hour.

Hitherto lack of knowledge of the nature of plant auximones has retarded research, but it is hoped that, just as the investigation of "deficiency" diseases was promoted by Eykman's* production of polyneuritis in birds, and the inducement of scurvy in guinea-pigs by Fürst†, the discovery of a bacterial test will facilitate an examination of the occurrence, nature, and composition of plant auximones.

* Eykman, 'Virchow's Arch.,' vol. 148, p. 523 (1897).

† Fürst, 'Verh. des 6 Nord. Kongress f. Inn. Med.,' p. 342 (1909).